

University of Groningen

## **Integrated Genomics of Crohn's Disease Risk Variant Identifies a Role for CLEC12A in Antibacterial Autophagy**

Begun, Jakob; Lassen, Kara G.; Jijon, Humberto B.; Baxt, Leigh A.; Goel, Gautam; Heath, Robert J.; Ng, Aylwin; Tam, Jenny M.; Kuo, Szu-Yu; Villablanca, Eduardo J.

*Published in:*  
Cell reports

*DOI:*  
[10.1016/j.celrep.2015.05.045](https://doi.org/10.1016/j.celrep.2015.05.045)

**IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.**

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2015

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Begun, J., Lassen, K. G., Jijon, H. B., Baxt, L. A., Goel, G., Heath, R. J., Ng, A., Tam, J. M., Kuo, S.-Y., Villablanca, E. J., Fagbami, L., Oosting, M., Magadi Gopalaiah, V. K., Schenone, M., Carr, S. A., Joosten, L. A. B., Vyas, J. M., Daly, M. J., Netea, M. G., ... Xavier, R. J. (2015). Integrated Genomics of Crohn's Disease Risk Variant Identifies a Role for CLEC12A in Antibacterial Autophagy. *Cell reports*, 11(12), 1905-1918. <https://doi.org/10.1016/j.celrep.2015.05.045>

### **Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

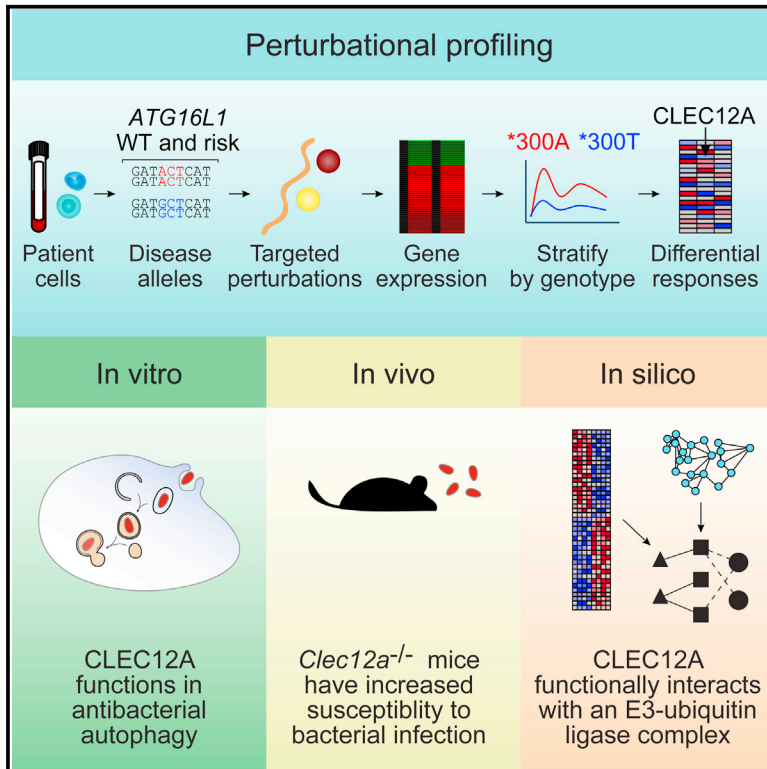
### **Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

# Integrated Genomics of Crohn's Disease Risk Variant Identifies a Role for CLEC12A in Antibacterial Autophagy

## Graphical Abstract



## Authors

Jakob Begun, Kara G. Lassen, Humberto B. Jijon, ..., Gordon D. Brown, Cisca Wijmenga, Ramnik J. Xavier

## Correspondence

klassen@broadinstitute.org (K.G.L.), xavier@molbio.mgh.harvard.edu (R.J.X.)

## In Brief

Although genome-wide association studies are valuable in identifying disease-associated loci, they produce only a partial view of pathogenesis. Using integrated, systems-level approaches to pinpoint genes that interact with the Crohn's-disease-associated variant *ATG16L1* T300A, Begun et al. identify *CLEC12A* as an innate defense gene that functions in antibacterial autophagy.

## Highlights

- Integrated genomics reveals risk-allele-specific autophagy pathway interactions
- *CLEC12A* is important for antibacterial autophagy in epithelial and immune cells
- *CLEC12A* knockdown amplifies antibacterial autophagy defects in *ATG16L1* \*300A cells
- *Clec12a*<sup>-/-</sup> mice are more susceptible to *Salmonella* infection in vivo

## Accession Numbers

GSE68765  
GSE69132



# Integrated Genomics of Crohn's Disease Risk Variant Identifies a Role for CLEC12A in Antibacterial Autophagy

Jakob Begun,<sup>1,2,3,12</sup> Kara G. Lassen,<sup>2,4,12,\*</sup> Humberto B. Jijon,<sup>1,2,5</sup> Leigh A. Baxt,<sup>2</sup> Gautam Goel,<sup>2</sup> Robert J. Heath,<sup>2,4</sup> Aylwin Ng,<sup>1,2,4</sup> Jenny M. Tam,<sup>6,7</sup> Szu-Yu Kuo,<sup>4</sup> Eduardo J. Villablanca,<sup>1,2,4,13</sup> Lola Fagbami,<sup>4,14</sup> Marije Oosting,<sup>8</sup> Vinod Kumar,<sup>9</sup> Monica Schenone,<sup>4</sup> Steven A. Carr,<sup>4</sup> Leo A.B. Joosten,<sup>8</sup> Jatin M. Vyas,<sup>6,7</sup> Mark J. Daly,<sup>4,10</sup> Mihai G. Netea,<sup>8</sup> Gordon D. Brown,<sup>11</sup> Cisca Wijmenga,<sup>9</sup> and Ramnik J. Xavier<sup>1,2,4,\*</sup>

<sup>1</sup>Gastrointestinal Unit and Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital, Boston, MA 02114, USA

<sup>2</sup>Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, MA 02114, USA

<sup>3</sup>Mater Research Institute, University of Queensland, Brisbane, QLD 4101, Australia

<sup>4</sup>The Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA

<sup>5</sup>Division of Gastroenterology, Department of Medicine, University of Calgary, Calgary, AB T2N 4N1, Canada

<sup>6</sup>Division of Infectious Diseases, Department of Medicine, Massachusetts General Hospital, Boston, MA 02114, USA

<sup>7</sup>Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

<sup>8</sup>Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Nijmegen Medical Center, Nijmegen 6525 GA, the Netherlands

<sup>9</sup>Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen 9700 RB, the Netherlands

<sup>10</sup>Analytic and Translational Genetics Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

<sup>11</sup>Aberdeen Fungal Group, Division of Applied Medicine, CLSM, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

<sup>12</sup>Co-first author

<sup>13</sup>Present address: Translational Immunology Unit, Department of Medicine, Solna, Karolinska Institutet and University Hospital, 171 77 Stockholm, Sweden

<sup>14</sup>Present address: Chemical Biology Program, Harvard Medical School, Boston, MA 02115, USA

\*Correspondence: [klassen@broadinstitute.org](mailto:klassen@broadinstitute.org) (K.G.L.), [xavier@molbio.mgh.harvard.edu](mailto:xavier@molbio.mgh.harvard.edu) (R.J.X.)

<http://dx.doi.org/10.1016/j.celrep.2015.05.045>

This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## SUMMARY

The polymorphism *ATG16L1* T300A, associated with increased risk of Crohn's disease, impairs pathogen defense mechanisms including selective autophagy, but specific pathway interactions altered by the risk allele remain unknown. Here, we use perturbational profiling of human peripheral blood cells to reveal that *CLEC12A* is regulated in an *ATG16L1*-T300A-dependent manner. Antibacterial autophagy is impaired in *CLEC12A*-deficient cells, and this effect is exacerbated in the presence of the *ATG16L1*\*300A risk allele. *Clec12a*<sup>−/−</sup> mice are more susceptible to *Salmonella* infection, supporting a role for *CLEC12A* in antibacterial defense pathways in vivo. *CLEC12A* is recruited to sites of bacterial entry, bacteria-autophagosome complexes, and sites of sterile membrane damage. Integrated genomics identified a functional interaction between *CLEC12A* and an E3-ubiquitin ligase complex that functions in antibacterial autophagy. These data identify *CLEC12A* as early adaptor molecule for antibacterial autophagy and highlight perturbational profiling as a method to elucidate defense pathways in complex genetic disease.

## INTRODUCTION

Genome-wide association studies (GWASs) have emerged as a powerful non-biased technique for identifying pathways related to human diseases with complex genetic architecture. Understanding such complex diseases requires a broad understanding of the relevant functional nodes regulating disease-relevant pathways. Genes do not function in isolation, and it is clear that both gene-gene and gene-environment interactions contribute to disease susceptibility (Barreiro et al., 2012; Cadwell et al., 2010; Silver et al., 2013). More than 140 genetic risk loci have been identified for Crohn's disease, a chronic inflammatory condition affecting the gastrointestinal tract (Franke et al., 2010; Jostins et al., 2012). These studies have highlighted the contribution of multiple immune and cellular pathways to Crohn's disease pathogenesis, particularly those involved in microbial defense (Jostins et al., 2012; Lassen et al., 2014; Murthy et al., 2014; Sadaghian Sadabad et al., 2014).

*ATG16L1* (autophagy-related protein 16-like 1) is a component of the core autophagy machinery, and the *ATG16L1* T300A polymorphism confers a modestly increased susceptibility to Crohn's disease despite its relatively high prevalence in the population (Franke et al., 2010). Autophagy is a pro-survival intracellular degradation pathway that functions as a key mediator of a number of processes including central metabolism, cell signaling, cell death, and carcinogenesis, as well as both innate and adaptive immunity (Deretic et al., 2013; Yang and

Klionsky, 2010). The *ATG16L1* T300A polymorphism has been associated with both impaired antibacterial autophagy and altered production of cytokines by peripheral blood mononuclear cells (PBMCs) in response to immune stimuli (Lassen et al., 2014; Plantinga et al., 2011). Recent studies have demonstrated that the mechanism underlying these alterations is linked to increased susceptibility of *ATG16L1* T300A to caspase-mediated cleavage and thus lower levels of functional *ATG16L1* (Lassen et al., 2014; Murthy et al., 2014).

The autophagy pathway is important for defense against a number of intracellular pathogens and pathobionts including *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, *Listeria monocytogenes*, *Enterococcus faecalis*, and *Mycobacterium tuberculosis* (Benjamin et al., 2013; Castillo et al., 2012; Gutierrez et al., 2004; Huett et al., 2012; Manzanillo et al., 2013; Ogawa et al., 2005; Tattoli et al., 2012; Thurston et al., 2012). Autophagy degrades intracellular bacteria and helps to restrict their replication. In this process, cytoplasmic bacteria are first marked for degradation primarily by ubiquitination (Deretic et al., 2013). Recent studies have identified key roles for the E3 ligases LRSAM1 and PARKIN in recognition and ubiquitination of cytoplasmic bacteria; however, there are likely additional cellular E3 ligases that provide specificity to this pathway (Huett et al., 2012; Manzanillo et al., 2013). Ubiquitinated bacteria are then recognized by the adaptor proteins p62, optineurin, and NDP52, leading to the recruitment of LC3 and the autophagy machinery (Cemna et al., 2011; Thurston et al., 2009; Wild et al., 2011). The autophagy pathway targets only a subpopulation of bacteria within a cell, and little is known about the molecules involved in the selective recognition and targeting of bacteria for autophagosomal degradation, despite the important pathological consequences of this process.

In this study, we approached the question of the impact of the *ATG16L1* T300A risk variant by searching for associated responsiveness quantitative trait loci (reQTLs), which are defined as the effects of genetic variation on transcriptional responses of cells (Barreiro et al., 2012). Given that functionally similar genes are often transcriptionally coregulated, using multiple immune stimuli in genetically defined cell populations along with reQTLs has enabled the identification of differential effects on molecular circuits (Gat-Viks et al., 2013). In the current study, we perform stimulus-specific perturbational profiling in PBMCs, quantitative mass-spectrometry-based (MS) proteomics, and genome-wide RNA expression analysis to identify antibacterial genes that are functionally related to *ATG16L1*. Using these approaches, we demonstrate a role for CLEC12A in antibacterial autophagy and pinpoint a functionally relevant interaction node of CLEC12A with an E3 ligase complex that is important for this function.

## RESULTS

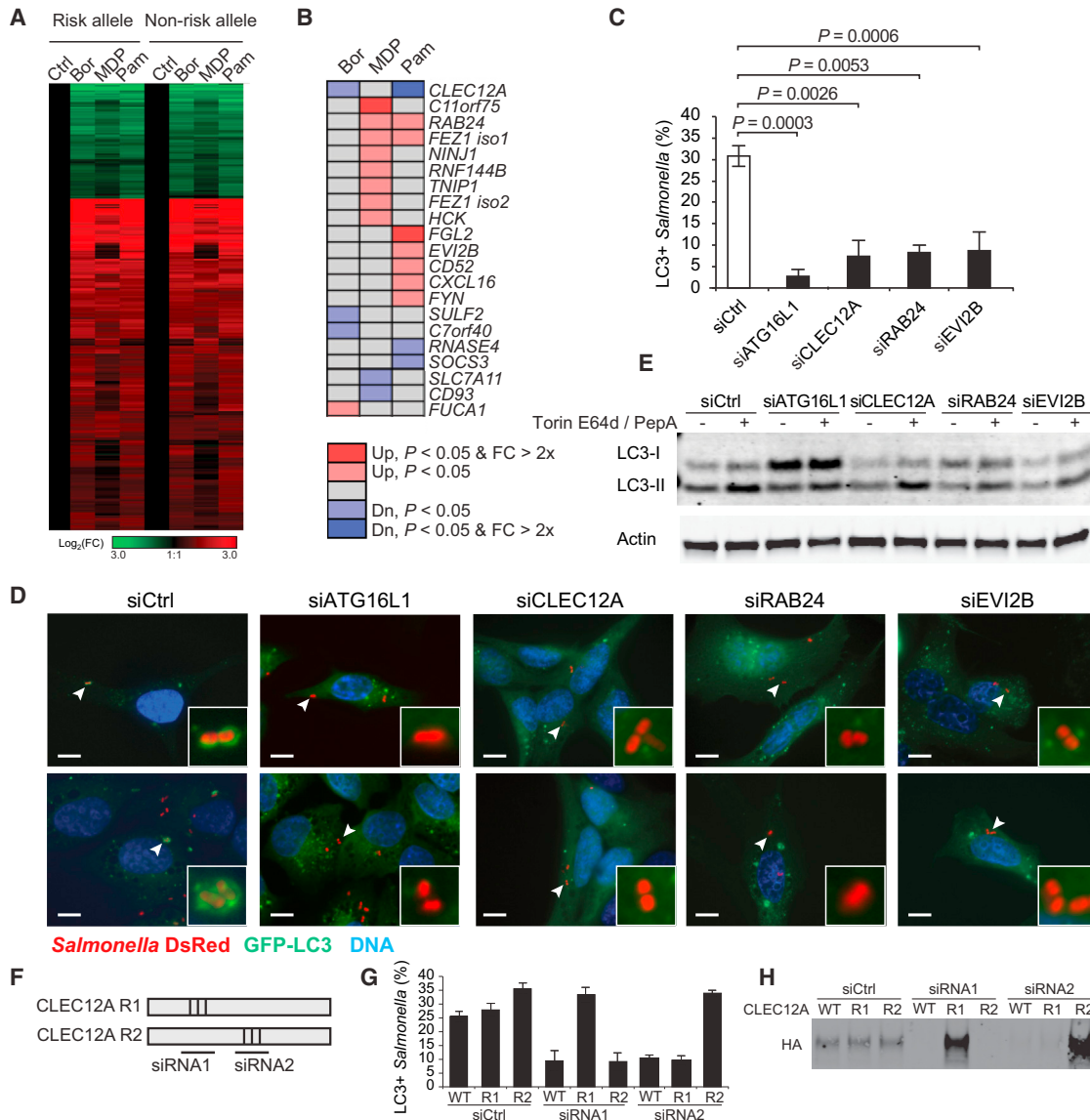
### Perturbational Profiling Reveals Genotype-Specific Transcriptional Responses

To identify molecules involved in the *ATG16L1*-T300A-dependent antibacterial autophagy pathway, we used perturbational profiling to measure differential gene regulation in the setting of stimulation with pathogen-associated molecular patterns and bacterial challenge. Given that mounting evidence indicates an

important role for the involvement of host-microbiota interactions and bacterial defense pathways in Crohn's disease (Knights et al., 2013; Lassen et al., 2014; Murthy et al., 2014), we examined cellular responses triggered upon exposure to various bacterial products. We selected a panel of three stimuli to perturb PBMCs from healthy individuals homozygous for the ancestral *ATG16L1*\*300T allele or the \*300A risk allele (rs2241880). The NOD2 ligand muramyl dipeptide (MDP) was selected based on the previous association of this stimulus with Crohn's-disease-specific pathways (Hugot et al., 2001; Inohara et al., 2003; Ogura et al., 2001). MDP can also modulate signals mediated through TLR2 activation, a pathway that has been implicated in Crohn's disease and experimental colitis (Netea et al., 2004; Watanabe et al., 2008). We therefore also used the TLR2 ligand Pam<sub>3</sub>Cys, as well as the bacterium *Borrelia burgdorferi*, from which these lipopeptides were initially isolated, to elicit both NOD2-dependent and -independent responses. Using microarrays to assess genome-wide RNA expression profiles, we found that baseline gene expression was similar between individuals harboring the non-risk and risk alleles. Furthermore, upon exposure to innate immune ligands, we observed strong genotype-independent induction of proinflammatory cytokines and chemokines after hierarchical clustering (Figures 1A and S1A). To identify reQTLs (Barreiro et al., 2012), we next used factorial design analysis to examine whether transcriptional responses to Pam<sub>3</sub>Cys, *Borrelia*, or MDP differed between groups stratified by the \*300T non-risk and \*300A risk alleles. Using multiple immune stimuli in genetically defined PBMCs along with reQTLs has enabled differential effects on molecular circuits to be determined (Gat-Viks et al., 2013). Our analysis identified 20 *ATG16L1*\*300A-dependent reQTLs under at least one stimulation condition (Figures 1B and S1B). Thus, perturbational profiling of human immune cells revealed genotype-specific responses to stimulation.

### Multiple T300A-Dependent Differentially Regulated Genes Are Required for Autophagy Pathways

We focused additional analysis on the autophagy pathway, the cellular degradation system that has been implicated in risk of Crohn's disease by GWASs, which identified predisposing alleles in *ATG16L1* and *IRGM* (immunity-related GTPase family M) (Rioux et al., 2007). We and others have reported that the *ATG16L1* T300A polymorphism is associated with impaired antibacterial autophagy as well as altered production of cytokines by PBMCs in response to immune stimuli (Lassen et al., 2014; Murthy et al., 2014). Additionally, TLR stimulation, particularly through TLR2, has been shown to induce antibacterial autophagy (Anand et al., 2011; Delgado et al., 2008; Shin et al., 2010). To determine whether any of the identified genes might be associated with autophagy, we generated protein interaction networks anchored on core autophagy proteins as well as autophagy-associated components previously identified from a high-confidence interaction network derived from a systems-wide autophagy proteomics study (Behrends et al., 2010). Of the genes identified, *RAB24* was previously reported to have a direct effect on autophagy (Behrends et al., 2010; Munafó and Colombo, 2002), and three additional gene products (HCK, FEZ1, and FYN) interacted with the autophagy network via known protein-protein



**Figure 1. Perturbational Profiling Reveals Genotype-Specific Transcriptional Responses, Including Differentially Regulated Genes that Are Required for Autophagy**

(A) Gene expression analysis of PBMCs isolated from healthy individuals after infection or stimulation with *Borrelia burgdorferi* (Bor), MDP, or Pam<sub>3</sub>Cys (Pam). Heatmap shows log<sub>2</sub> (fold change) relative to mock-treated control.

(B) Heatmap identifying transcripts exhibiting response differences to infection or immune stimuli, stratified by ATG16L1 T300A polymorphism status.

(C) HeLa GFP-LC3 cells were transfected with indicated siRNAs and infected with DsRed-labeled *Salmonella*. The percentage of GFP-LC3+ *Salmonella* at 1 hr post-infection is shown. Data are shown as means  $\pm$  SD;  $n = 75$  cells per condition from three biological replicates. Data are representative of three independent experiments.

(D) Representative epifluorescent microscopy images of HeLa cells used to generate data shown in (C). The scale bars represent 10  $\mu$ m.

(E) Western blot of HeLa cells transfected with the indicated siRNAs and treated with Torin E64D/PepA. Data are representative of three independent experiments.

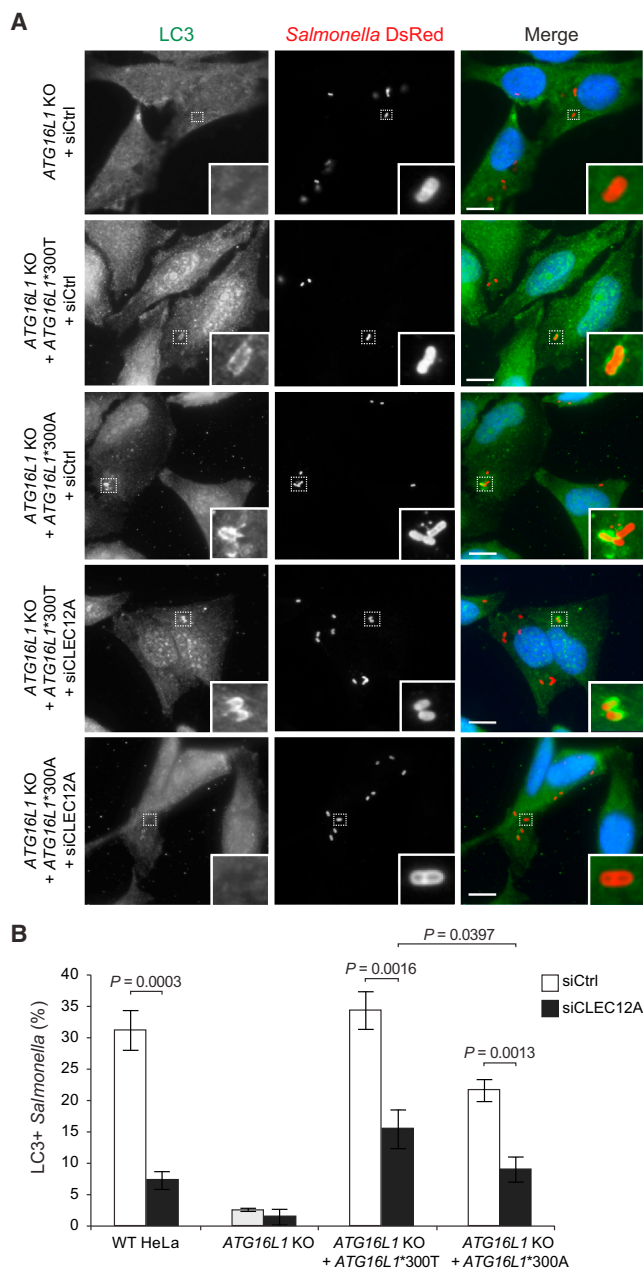
(F) CLEC12A rescue constructs indicating positions of synonymous mutations and location of targeting siRNA.

(G) HeLa GFP-LC3 cells were transfected with control siRNA, CLEC12A siRNA1, or CLEC12A siRNA2 as well as CLEC12A rescue constructs (R1 or R2) and infected for 1 hr with DsRed-labeled *Salmonella*. Data are shown as mean  $\pm$  SD;  $n = 75$  per condition. Data are representative of three independent experiments.

(H) HeLa cells from (G) were lysed, and HA-CLEC12A expression levels were assessed by western blot. Data are representative of three independent experiments.

See also Figure S1.





**Figure 2. CLEC12A Knockdown and the ATG16L1 T300A SNP Act Additively to Impair LC3-Bacteria Colocalization**

(A) Representative epifluorescent images showing impaired antibacterial autophagy in ATG16L1 KO HeLa cells complemented with the indicated ATG16L1 protein in the presence of control siRNA or CLEC12A siRNA. The scale bars represent 10  $\mu$ m.

(B) Percentage of LC3-Salmonella colocalization in WT HeLa cells, ATG16L1 KO HeLa cells, and ATG16L1 KO HeLa cells stably expressing ATG16L1\*300T or \*300A alleles in the presence of control siRNA or CLEC12A siRNA. Data are shown as means  $\pm$  SD; n = 75. Data shown are representative of three independent experiments.

See also Figure S2.

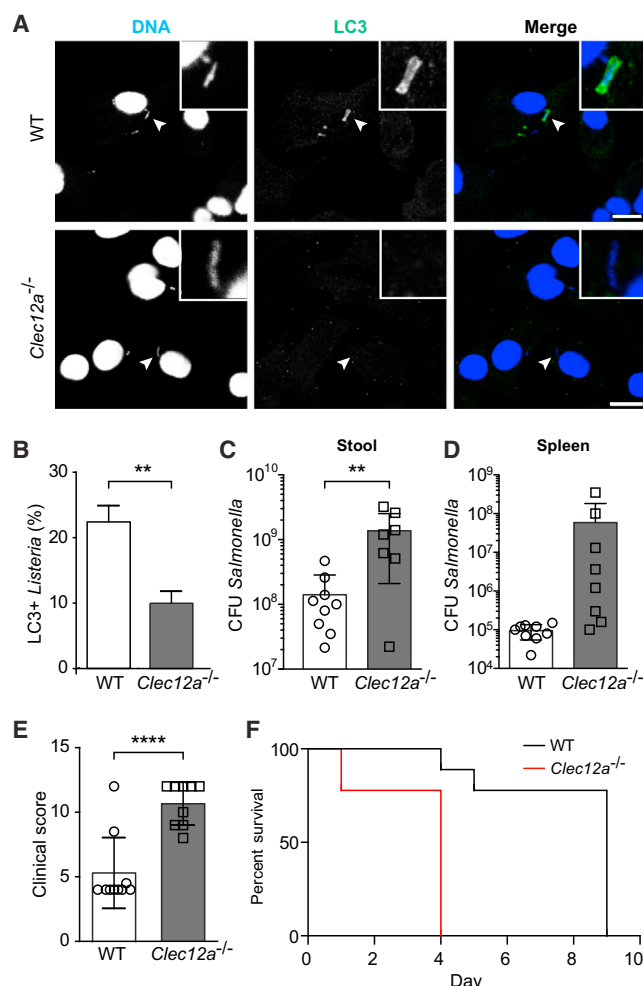
interactions (Figure S1C). These observations demonstrate that the ATG16L1 T300A polymorphism is associated with transcriptional modulation reflecting known protein-protein interactions with the autophagy network.

Given the previously described role for ATG16L1 and IRGM in antibacterial autophagy (Singh et al., 2006), we next used siRNA to evaluate whether any of the identified ATG16L1\*300A-dependent reQTLs affected antibacterial autophagy. Of the 20 genes identified, 13 had detectable expression in HeLa cells and were suppressed by single siRNAs (Figure S1D). Antibacterial autophagy was assessed in siRNA-treated cells using infection with *S. Typhimurium*, a model pathogen that is degraded by autophagy (Birmingham et al., 2006). Knockdown of three of these genes (CLEC12A, RAB24, and EVI2B) resulted in significantly decreased Salmonella-autophagosome colocalization (Figures 1C, 1D, and S1E).

We next tested whether RAB24, EVI2B, or CLEC12A play a role in classical autophagy using an LC3 flux assay, in which levels of lipidated LC3-II are compared to LC3-I by western blot. In this assay, an increase in the ratio of LC3-II to LC3-I corresponds to an increase in autophagic flux. Knockdown of RAB24 and EVI2 resulted in decreased LC3-II accumulation when cells were treated with Torin 1, an mTOR inhibitor and inducer of bulk autophagy, suggesting that these genes are involved in classical autophagy; knockdown of ATG16L1 served as a control in these experiments. Knockdown of CLEC12A did not impair LC3-II accumulation under these conditions, suggesting that CLEC12A functions specifically in antibacterial autophagy (Figure 1E) (Thoreen et al., 2009). Additionally, to confirm that the antibacterial autophagy phenotype seen with CLEC12A knockdown was not due to off-target effects, we employed a knockdown rescue approach (Huett et al., 2012) by generating two siRNA-resistant constructs that express full-length CLEC12A during knockdown of endogenous gene expression (Figure 1F). The autophagy defect caused by siRNA against CLEC12A was rescued by overexpression of the appropriate CLEC12A construct (Figures 1G and 1H), demonstrating that the decrease in antibacterial autophagy was specific to a reduction in CLEC12A expression and not due to off-target effects of siRNA. Additionally, siRNA targeting CLEC12A did not alter bacterial entry, suggesting the observed effect was specific to the autophagy pathway (Figure S1F).

### CLEC12A Knockdown and the ATG16L1 T300A SNP Act Additively to Impair LC3-Bacteria Colocalization

Given our findings that immune cells harboring the ATG16L1\*300A allele exhibited reduced induction of CLEC12A under immune stimulation conditions and that CLEC12A is required for efficient LC3-Salmonella colocalization (Figure 1), we next investigated whether a genetic interaction exists between the ATG16L1 T300A SNP and CLEC12A with respect to antibacterial autophagy. To investigate this hypothesis, we generated an ATG16L1 knockout (KO) HeLa cell line using the CRISPR-Cas9 system (Ran et al., 2013). Loss of ATG16L1 expression was confirmed in single HeLa clones (Figure S2A). As expected, ATG16L1 KO cells were unable to convert LC3-I to LC3-II under basal or stimulatory conditions (Figure S2B) and had negligible LC3-bacteria colocalization (Figure 2). To



**Figure 3. *Clec12a*<sup>-/-</sup> Mice Are More Susceptible to *Salmonella* Infection In Vivo**

(A) Confocal micrographs of LC3-*Listeria* colocalization in WT and *Clec12a*<sup>-/-</sup> BMDMs at 1 hr post-infection. The scale bars represent 10  $\mu$ m. (B) Quantification of LC3-*Listeria* colocalization in WT and *Clec12a*<sup>-/-</sup> BMDMs. Data shown represent mean  $\pm$  SD of  $n = 3$  independent experiments; unpaired t test. \*\* $p < 0.01$ . (C and D) *Salmonella* cfus quantified per gram of stool (C) and per organ (D) at 4 days post-infection are displayed. Data are shown as mean  $\pm$  SD; \*\* $p \leq 0.01$ . Data are representative of at least two independent experiments ( $n = 9$  for WT;  $n = 7$  or 8 for *Clec12a*<sup>-/-</sup>). (E) Clinical score for infected mice at 4 days post-infection. Data are shown as mean  $\pm$  SD. Data are representative of at least two independent experiments. \*\*\*\* $p < 0.0001$ ; unpaired t test. ( $n = 14$  for WT;  $n = 13$  for *Clec12a*<sup>-/-</sup>). (F) Survival curve for infected WT and *Clec12a*<sup>-/-</sup> mice ( $n = 9$  mice per genotype). See also Figure S3.

determine the effect of the T300A coding polymorphism, we stably transduced ATG16L1 KO cells with \*300T (ATG16L1 KO + ATG16L1\*300T) or \*300A (ATG16L1 KO + ATG16L1\*300A) alleles, achieving equivalent expression levels of ATG16L1 in these lines (Figure S2A). As expected, we found that basal autophagy was restored in both ATG16L1 KO + ATG16L1\*300T and ATG16L1 KO + ATG16L1\*300A cell lines, with modest

impairment of antibacterial autophagy in the ATG16L1 KO + ATG16L1\*300A cells, consistent with published findings (Figure 2) (Conway et al., 2013; Lassen et al., 2014; Murthy et al., 2014). Targeting CLEC12A with siRNA resulted in impaired antibacterial autophagy in the ATG16L1 KO + ATG16L1\*300T cell line similar to WT cells. We observed a more-pronounced impairment in ATG16L1 KO + ATG16L1\*300A cells, suggesting functional interaction of the T300A polymorphism and reduced CLEC12A expression.

### *Clec12a*<sup>-/-</sup> Mice Are More Susceptible to *Salmonella* Infection In Vivo

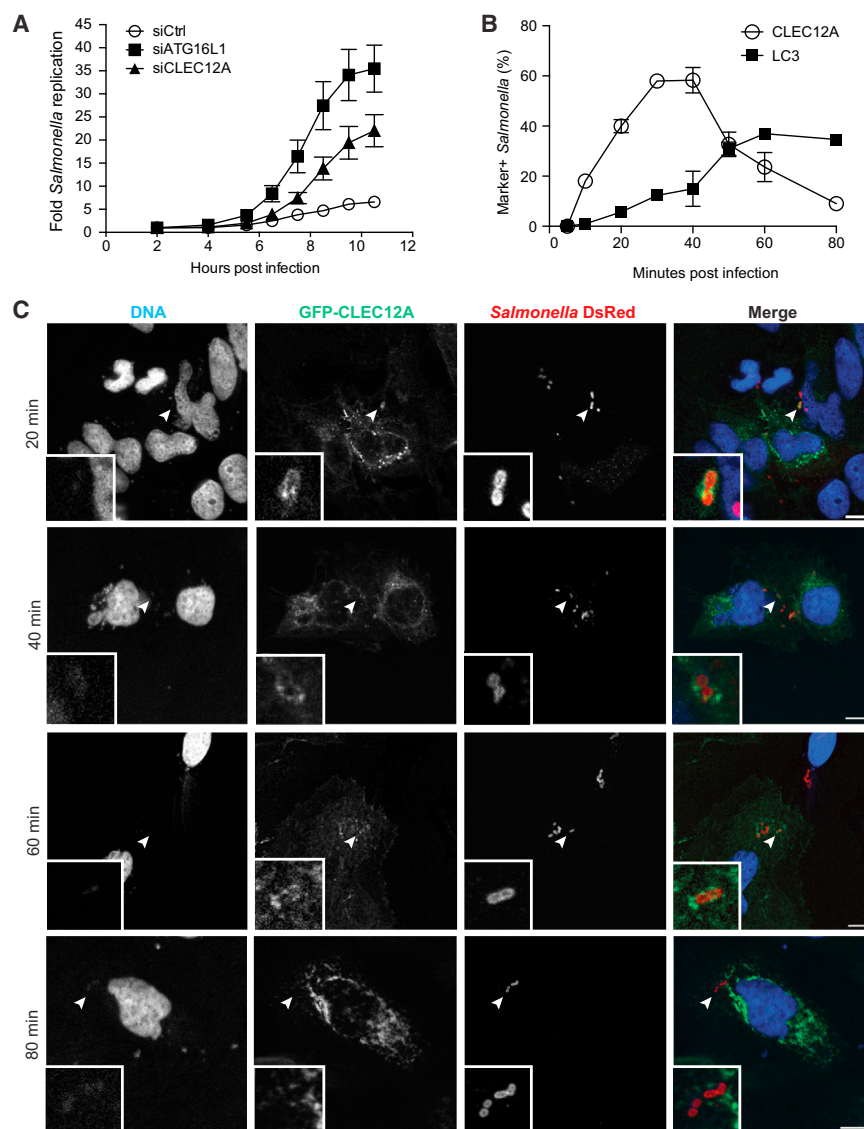
CLEC12A is a C-type lectin receptor (CLR) belonging to a family of transmembrane proteins that recognize pathogen-associated molecular patterns and engage downstream immune signal transduction pathways (Dambuzza and Brown, 2015; Osorio and Reis e Sousa, 2011). CLEC12A is highly expressed in human myeloid cells (Figure S3A), and targeting antigen to CLEC12A results in enhanced antigen presentation on dendritic cells in mice (Lahoud et al., 2009; Marshall et al., 2006). Recent studies have demonstrated that CLEC12A binds to uric acid crystals and helps to dampen inflammation through its ITIM motifs; however, it remains unclear whether this is the only ligand for CLEC12A (Neumann et al., 2014).

To determine whether CLEC12A plays a role in pathogen defense in vivo, we used *Clec12a*<sup>-/-</sup> mice. Bone-marrow-derived macrophages (BMDMs) from WT or *Clec12a*<sup>-/-</sup> mice were infected with a strain of *Listeria* (EGDe) known to be susceptible to autophagy in primary macrophages (Anand et al., 2011; Birmingham et al., 2007; Huett et al., 2012). Consistent with the *Salmonella* infection results in siRNA-treated cells, *Clec12a*<sup>-/-</sup> BMDMs displayed lower levels of bacterial colocalization with LC3 compared with WT BMDMs (Figures 3A and 3B). Additionally, *Clec12a*<sup>-/-</sup> BMDMs did not show differences in Torin-1-induced autophagy, suggesting that bulk autophagy is normal in these cells (Figure S3B). Taken together, these data suggest that CLEC12A functions selectively in the antibacterial autophagy pathway for multiple pathogens in both mouse and human cells.

We next investigated whether CLEC12A plays a role during infection with intracellular pathogens in vivo. WT or *Clec12a*<sup>-/-</sup> mice were pre-treated with streptomycin and then infected with *Salmonella* (Conway et al., 2013). Four days post-infection, stool was collected from infected mice and colony-forming units (cfus) were measured. *Clec12a*<sup>-/-</sup> mice had significantly higher cfu levels compared to WT mice (Figure 3C). *Clec12a*<sup>-/-</sup> mice also exhibited increased systemic bacterial dissemination as assessed by measuring cfus in spleens (Figure 3D). This increase in cfu was associated with a more-severe clinical disease score as assessed by overall appearance, piloerection, mobility, and posture (Figure 3E), as well as decreased survival (Figure 3F). Taken together, these data suggest that CLEC12A plays a role in intracellular microbial defense both in vitro and in vivo.

### CLEC12A Associates with Bacteria Early after Entry into Host Cells and Restricts Intracellular Bacterial Replication

To further demonstrate the precise role for CLEC12A in antibacterial autophagy, we next tested whether knockdown of



**Figure 4. CLEC12A Associates with Bacteria Early after Entry into Host Cells and Restricts Intracellular Bacterial Replication**

(A) Gentamicin protection assay in HeLa cells showing fold replication of *Salmonella* in cells treated with the indicated siRNAs. Data are shown as mean  $\pm$  SD;  $n = 6$ . Data are representative of four independent experiments.

(B) Quantitation of CLEC12A-bacteria and LC3-bacteria colocalization at indicated time points. Data are shown as mean  $\pm$  SD;  $n = 3$  independent experiments.

(C) Representative confocal micrographs of HeLa cells showing association of intracellular bacteria with GFP-CLEC12A at 20, 40, 60, and 80 min post-infection. The scale bars represent 10  $\mu$ m.

See also Figure S4.

(Figure S4B), consistent with the time course of peak LC3 colocalization. Given the early association of CLEC12A with bacteria, we next determined whether CLEC12A was present at sites of bacterial entry into the host cell. We found that GFP-CLEC12A colocalized with bacteria and ruffled actin, an indicator of recent bacterial entry, suggesting that CLEC12A associates with bacteria concomitant with bacterial cellular entry (Figure S4C) (Lhocine et al., 2015).

#### CLEC12A Colocalizes with Autophagosome Proteins

Next, we investigated whether CLEC12A colocalizes with markers of antibacterial autophagy. We confirmed that CLEC12A colocalizes with *Salmonella* and Rab5, a marker of early endosomes (Figure S5A). However, CLEC12A recruitment was also readily detected around *Salmonella* without colocalization of Rab5, suggest-

ing that CLEC12A is not exclusively associated with endosomes (Figure S5A). Next, we evaluated the colocalization of CLEC12A with galectin 3 and galectin 8, which are both known to bind to sites of bacterial-induced intracellular membrane damage as well as sites of sterile membrane damage (Thurston et al., 2012). CLEC12A colocalized with both galectin 3- and galectin 8-*Salmonella* complexes (Figure 5A). These data confirm that CLEC12A associates with bacteria early after infection concomitant with membrane damage.

CLEC12A altered intracellular bacterial replication. Using knock-down of *ATG16L1* as a positive control, we found that knock-down of *CLEC12A* in HeLa cells resulted in significantly increased intracellular replication of *Salmonella* (Figure 4A), demonstrating that CLEC12A is required for restriction of intracellular bacterial replication.

Next, we determined whether CLEC12A localizes to bacteria within cells. GFP-CLEC12A (Figures 4B and 4C) and endogenous CLEC12A (Figure S4A) were predominantly membrane associated and could be found surrounding intracellular DsRed-labeled *Salmonella* at both early and late time points after infection. Association of CLEC12A with *Salmonella* peaked at 30 min post-infection and declined gradually, whereas LC3-*Salmonella* colocalization peaked 60 min after infection, consistent with previous data (Figure 4B) (Huett et al., 2012). Live cell imaging confirmed that GFP-CLEC12A surrounds bacteria and then dissociates at approximately 1 hr post-infection

ing that CLEC12A is not exclusively associated with endosomes (Figure S5A). Next, we evaluated the colocalization of CLEC12A with galectin 3 and galectin 8, which are both known to bind to sites of bacterial-induced intracellular membrane damage as well as sites of sterile membrane damage (Thurston et al., 2012). CLEC12A colocalized with both galectin 3- and galectin 8-*Salmonella* complexes (Figure 5A). These data confirm that CLEC12A associates with bacteria early after infection concomitant with membrane damage.

CLEC12A also colocalized with the antibacterial autophagy adaptor NDP52 (Figure 5B) as well as the autophagosome proteins GFP-LC3, the ATG8 homolog GABARAPL2, and ATG16L1 (Figures 5C and 5D). In all cases, these proteins surrounded intracellular bacteria in conjunction with either endogenous CLEC12A or GFP-CLEC12A (Figures 5B–5D). Of note, expression of GFP-CLEC12A gave a more-intense fluorescent signal and demonstrated increased levels of CLEC12A puncta



within the cells, regardless of stimulation or bacterial infection. Physical interactions between CLEC12A and LC3, GABARAPL2, and ATG16L1 were also confirmed by co-immunoprecipitation (Figure S5B). No difference was observed in CLEC12A binding to ATG16L1\*300T compared to \*300A.

Next, we investigated the importance of CLEC12A to early stages in bacterial targeting. We first determined whether knockdown of *CLEC12A* altered recruitment of ubiquitin to bacteria. At 1 hr post-infection, siRNA knockdown of *CLEC12A* significantly reduced *Salmonella*-ubiquitin colocalization (Figures 5E and 5F). Consistent with this observation, recruitment of NDP52 to *Salmonella* was also decreased upon siRNA knockdown of CLEC12A (Figures 5G and 5H). Colocalization of *Salmonella* with galectin 3, galectin 8, and p62 were unchanged in cells treated with CLEC12A siRNA (Figures S5C–S5E). These data reveal CLEC12A as an early bacteria-associated factor that binds to the autophagy machinery and helps initiate antibacterial autophagy through the ubiquitin-NDP52 pathway.

Recently, CLEC12A has been shown to be a receptor for dead cells (Neumann et al., 2014). We hypothesized that CLEC12A could also recognize sites of membrane damage triggered by vacuolar disruption after bacterial entry (Tattoli et al., 2012; Thurston et al., 2012). To test this hypothesis, we triggered osmotic damage of endosomes in cells expressing GFP-CLEC12A. Galectin 3 served as a marker of damaged endosomes (Thurston et al., 2012). GFP-CLEC12A colocalized with galectin 3 puncta after hypertonic shock and treatment with polyethylene glycol (PEG) (Figure 5I). These results demonstrate that CLEC12A is recruited to sites of membrane damage and could explain the high proportion of CLEC12A recruitment early after bacterial entry.

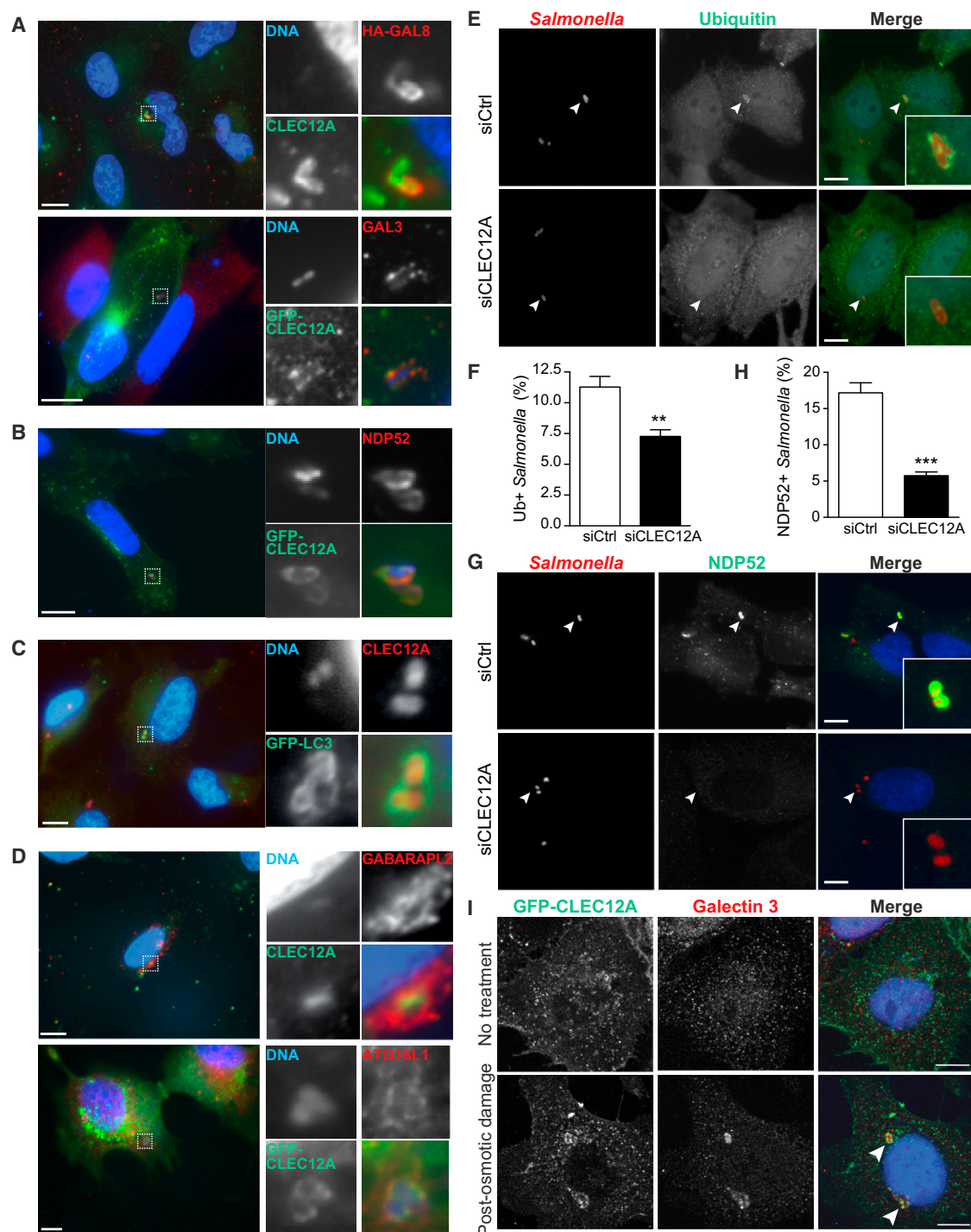
### Integrated ‘Omics Analysis Identifies KLHL9, KLHL13, and NEDD8 as CLEC12A-Interacting Antibacterial Autophagy Molecules

To develop a more-integrated model of CLEC12A function in pathogen response, we utilized time course RNA-seq data and targeted quantitative proteomics to pinpoint CLEC12A-dependent protein interactions that may be important for pathogen response. First, we employed 3' RNA-seq to quantify the transcriptome of *Listeria*-infected WT or *Clec12a*<sup>−/−</sup> BMDMs at various times post-infection. Using this approach, we identified genes with statistically significant differences (FDR-adjusted  $q$  value  $\leq 0.05$ ) in fold-change response to *Listeria* infection between WT and *Clec12a*<sup>−/−</sup> BMDMs at any time point post-infection (Figure 6A). Of note, expression of LAMP1, a lysosomal membrane protein and marker of *Salmonella*-containing vacuoles, was significantly downregulated in *Clec12a*<sup>−/−</sup> BMDMs, suggesting endomembrane dynamics might be perturbed in these cells. We next performed clustering and pathway analysis using an expanded list of 674 nominally significant genes that were differentially regulated in *Clec12a*<sup>−/−</sup> BMDMs at baseline as well as in response to *Listeria*. Genes were grouped into ten clusters using Short Time-series Expression Miner (STEM), where each cluster represents one of the predefined model profiles in STEM that capture potential distinct patterns of infection response (Figure 6B; Table S1) (Ernst and Bar-Joseph, 2006). Differences in these model profiles represent distinct regulation patterns of gene expression. Gene ontology enrichment analysis

was applied to assess the functional significance of each cluster of genes. The top scoring pathway that was differentially regulated in *Clec12a*<sup>−/−</sup> BMDMs was the negative regulation of the intracellular signal transduction pathway that includes key signaling components such as *Ndufs3*, *Tmem161a*, and *Mapkapk5*, among others (cluster 2; Table S1). Additionally, anti-fungal response genes (*S100a9*, *Myd88*, and *Ptx3*; cluster 6) were also differentially regulated in the *Clec12a*<sup>−/−</sup> BMDMs. Selective upregulation of glucose transport (*Pbbp* and *Sorbs1*; cluster 7) but downregulation of lipid transport (*Scarb1*, *Slimo1*, and *Soat2*; cluster 5) in *Clec12a*<sup>−/−</sup> cells suggested preferential regulation of metabolism by CLEC12A in response to *Listeria* infection. These results highlight the central role of CLEC12A in pathogen-induced defense pathways and suggest how deficiencies in CLEC12A could result in impaired bacterial defense.

Next, we sought to identify important transcriptional nodes that control these responses by integrating known transcription factor-DNA interaction data with our temporal gene expression profiles of infected WT and *Clec12a*<sup>−/−</sup> BMDMs into a unified model. To identify important regulation points in the CLEC12A pathway, we used Dynamic Regulatory Events Miner (DREM), which searches for CLEC12A-dependent bifurcation events, defined by a transition in which a set of genes that were previously coregulated instead show divergent response/regulation profiles (Figure S6A) (Schulz et al., 2012). Based on an extension of a hidden Markov model, the analysis identified EGR1, a transcription factor involved in transcriptional response to pathogens (de Grado et al., 2001; McDermott et al., 2011), as the earliest stage regulator that induces CLEC12A-dependent downregulation of specific genes (Figure S6A). Interestingly, EGR1 is also known to be induced by either amino acid deprivation or ER stress, which are both stimuli known to trigger autophagy (Shan et al., 2014). These data suggest that reduced expression of EGR1 target genes could contribute to the reduced pathogen response and autophagic targeting of bacteria in *Clec12a*<sup>−/−</sup> BMDMs. Other notable transcriptional regulators identified in our analysis that are known to be important for pathogen response include RELA, NFE2, HIF1A, and ARNT2. Taken together, this analysis suggests that deletion of *Clec12a* results in an alteration of the response to pathogens at the transcriptional level and identifies key regulators involved in this response.

To further understand the role of CLEC12A in antibacterial autophagy, we next applied an integrated systems approach using quantitative MS-based proteomics and coupled these results with our transcriptomic profiling analysis to uncover functional CLEC12A interactions relevant to bacterial defense (Figure 6C). Proteomic interactors were defined as those that demonstrated significantly increased binding to FLAG-CLEC12A relative to FLAG vector alone after immunoprecipitation (Table S2). Interactome analysis identified highly connected nodes within the CLEC12A interactors (Figure S6B). A specific interaction between CLEC12A and LC3 or ATG16L1 was not identified by MS-based proteomics; however, this may reflect the fact that a meaningful interaction between these proteins occurs only under stimulation and represents a fraction of the total cellular pool of these proteins. To identify CLEC12A protein interactions that may lead to the measured transcriptomic changes in response to bacterial infection, we used a network optimization approach



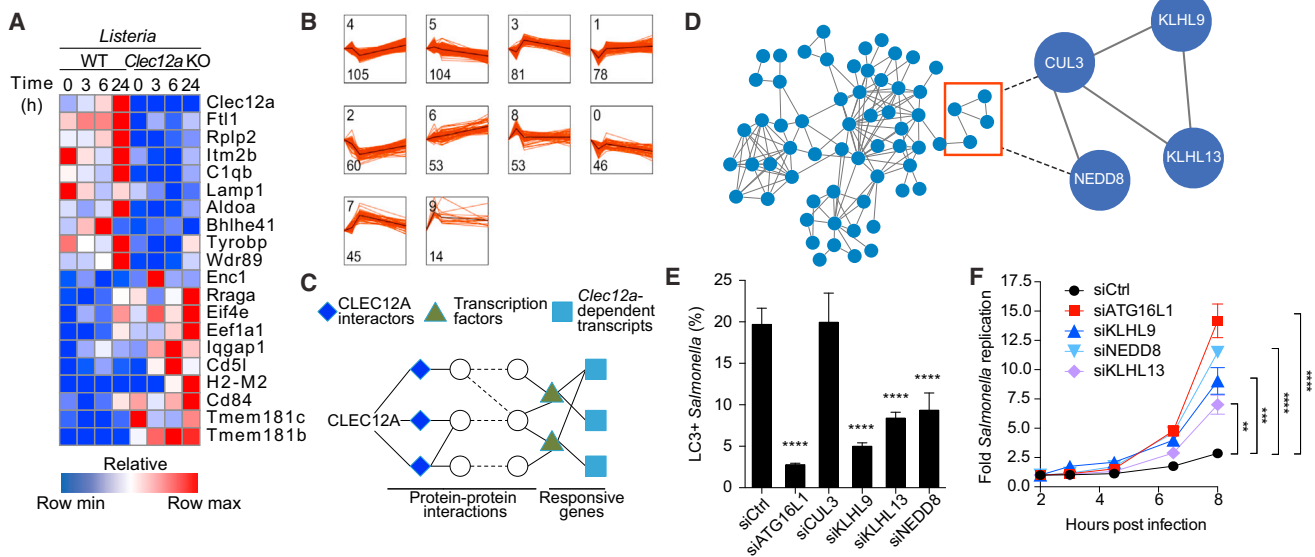
**Figure 5. CLEC12A Colocalizes with Autophagosome Proteins and Plays a Role in Pathogen Defense**

(A–C) Representative epifluorescent images of HeLa cells showing colocalization of (A) CLEC12A (green) and galectin 8 or galectin 3 (red) around intracellular *Salmonella* (blue), (B) GFP-CLEC12A (green) and NDP52 (red) around *Salmonella* (blue), and (C) endogenous CLEC12A (red) and GFP-LC3 (green) around *Salmonella* (blue).

(D) CLEC12A or GFP-CLEC12A (green) and GABARAPL2 or ATG16L1 (red) around intracellular *Salmonella* (blue). Images are representative of three or more independent experiments.

(E) Representative epifluorescent images of HeLa cells transfected with a control siRNA or a siRNA against *CLEC12A* and infected with DsRed-labeled *Salmonella* for 1 hr.

(legend continued on next page)



**Figure 6. Integrated Systems Analysis Identifies KLHL9, NEDD8, and KLHL13 as CLEC12A-Interacting Antibacterial Autophagy Molecules**  
(A) Gene expression analysis of WT and *Clec12a*<sup>-/-</sup> BMDMs infected with *Listeria* for the indicated times. Heatmap shows log<sub>2</sub> (fold change) relative to uninfected WT BMDMs at indicated time points. Shown are the top ten most-significant upregulated and downregulated genes in *Clec12a*<sup>-/-</sup> BMDMs compared to WT BMDMs.

(B) Cluster analysis of 674 differentially expressed genes in *Clec12a*<sup>-/-</sup> BMDMs versus WT BMDMs.

(C) Schematic of integrated proteomic and transcriptomic approach to identify functionally relevant CLEC12A-interacting proteins.

(D) Interactome analysis shows network of proteins identified from quantitative iTRAQ proteomics. Red box highlights the KLHL9-KLHL13-CUL3-NEDD8 complex interaction within the CLEC12A interactome.

(E) HeLa GFP-LC3 cells were transfected with indicated siRNAs for 48 hr and then infected with DsRed-labeled *Salmonella* for 1 hr. The fraction of GFP-positive intracellular *Salmonella* is shown. Data are mean ± SD and are representative of three independent experiments.

(F) Gentamicin protection assay in HeLa cells showing increased intracellular bacterial replication after 48 hr of knockdown of *ATG16L1*, *KLHL9*, *NEDD8*, or *KLHL13*. Data shown as mean ± SD; n = 4. Data are representative of three independent experiments.

See also Figure S6 and Tables S1, S2, and S3.

to identify high-probability sub-networks, composed of signaling and transcription factor interaction cascades (Figure 6C) (Basha et al., 2013). We then used a hypergeometric test to rank order CLEC12A-interacting proteins with the most significantly enriched set of target genes within the network to identify relevant functional nodes (Table S3). Some of the key transcriptional regulators identified in these sub-networks overlapped with those identified by DREM analysis, including RELA, TP53, HIF1A, and EGR1. This analysis pinpointed the ubiquitin-like protein, NEDD8 (neural precursor cell expressed, developmentally downregulated 8), as both a CLEC12A interactor and a regulator of EGR1. These results suggest that the interaction between CLEC12A and NEDD8 is a functional interaction and contributes to an early CLEC12A-dependent transcriptional response. This integrated approach also linked an E3 ubiquitin ligase complex

that includes CUL3 (cullin 3) and the substrate adaptors KLHL9 (kelch-like family member 9) and KLHL13 (kelch-like family member 13) to CLEC12A-dependent transcriptional responses controlled by the transcription factors CEBPB and CEBPD (Table S3) (Chen et al., 2014). Importantly, proteomic interactome analysis demonstrated a physical interaction between NEDD8, KLHL13, CUL3, and KLHL9, indicating that this is a functional E3 ligase complex that interacts with CLEC12A (Figure 6D).

NEDD8 is a target of some cycle-inhibiting factors (Cifs), which are virulence factors produced by bacterial pathogens that alter the host cell cycle (Crow et al., 2012; Jubelin et al., 2010). Cif can associate directly with NEDD8-modified cullin-RING complexes and inhibit their activity (Crow et al., 2012; Jubelin et al., 2010). These results suggest that these E3 complexes could function to restrict bacterial replication. We therefore investigated

(F) Quantitation of ubiquitin-bacteria colocalization at 1 hr post-infection in cells shown in (E). Data are shown as means ± SEM from three independent experiments.

(G) Representative epifluorescent images of HeLa cells transfected and infected as in (E).

(H) Quantitation of NDP52-bacteria colocalization at 1 hr post-infection in cells shown in (G). Data are shown as means ± SEM from three independent experiments.

(I) Representative confocal micrographs of HeLa cells expressing GFP-CLEC12A. Cells were untreated or exposed to hypertonic media containing PEG 1000, followed by hypotonic shock.

All scale bars represent 10 μm. See also Figure S5.



whether CUL3, NEDD8, KLHL9, and KLHL13 had roles in antibacterial autophagy similar to CLEC12A (Huett et al., 2012; Manzanillo et al., 2013). Upon siRNA knockdown in HeLa cells, KLHL9, KLHL13, and NEDD8 had a dramatic effect on both LC3-*Salmonella* colocalization and bacterial replication, consistent with a role for these proteins in antibacterial autophagy (Figures 6E and 6F).

## DISCUSSION

Human genetics provides a powerful tool to identify and understand physiologically relevant host responses to pathogens given that host-pathogen interactions are known to have a role in many complex diseases. Gene-gene interactions and environment-gene interactions add to the complexity of identifying causal genes and variants from GWAS data, as well as determining how these variants affect pathogenesis. Pathway-specific perturbational profiling and integrated systems approaches have previously been used in genetically defined organisms to identify genetic nodes of regulation underlying disease (Gagneur et al., 2013; Gat-Viks et al., 2013; Smith and Kruglyak, 2008; Zhang et al., 2013). Using this strategy, we uncovered bacteria- and bacterial-ligand-specific programs affected in healthy individuals homozygous for the T300A polymorphism in the autophagy gene *ATG16L1*. Interestingly, 15% of the T300A-dependent response genes were found to play a role in autophagy pathways, highlighting important SNP-pathway interactions.

Our network analysis identified the CLR family member *CLEC12A* as a gene that is less responsive upon TLR2 or bacterial stimulation in individuals carrying the *ATG16L1* \*300A SNP. Pursuing the functional implication of this SNP-environment interaction, we identified a role for *CLEC12A* in antibacterial autophagy and demonstrated that *CLEC12A* controls microbial replication in vitro and in vivo. Using cells engineered to express the Crohn's disease non-risk or risk allele of *ATG16L1*, we interrogated the SNP-gene interaction with *CLEC12A* to demonstrate an exacerbated effect on antibacterial autophagy in the setting of the disease-associated SNP. Importantly, knockdown of *CLEC12A* altered both ubiquitin-*Salmonella* colocalization and NDP52-*Salmonella* colocalization. These findings are consistent with *CLEC12A* functioning at an early step in the antibacterial autophagy pathway at the level of pathogen recognition caused by membrane damage, with *ATG16L1* functioning downstream at the level of autophagosome formation. These data suggest a model in which individuals who carry the \*300A risk allele have reduced *CLEC12A* responsiveness that serves to compound defects in antibacterial autophagy. These results highlight how non-redundant genes in the same pathway can be identified through ligand-specific transcriptional regulation.

CLRs are a large family of pattern recognition receptors, many of which are important signaling mediators in antimicrobial defense (Drummond and Brown, 2013). *CLEC12A* was recently shown to be a receptor for uric acid crystals and dead cells (Neumann et al., 2014), and we extend this finding to demonstrate that *CLEC12A* also recognizes sites of intracellular membrane damage triggered by bacterial entry (Tattoli et al., 2012; Thurston et al., 2012). Additionally, we show that *CLEC12A* likely serves as an adaptor to help recruit ubiquitin

and NDP52 to sites of vacuolar damage caused by bacteria and thus induce antibacterial autophagy. These data suggest parallels between the functions of galectins and CLRs as early sensors of damage in selective autophagy pathways. Additionally, a dendritic cell CLR, DNGR-1, regulates endocytic handling of necrotic cell antigens to modulate cross-priming, suggesting that CLRs could function broadly in the selection of endocytic cargo (Zelenay et al., 2012). Recent data also indicate a role for another CLR, CLEC16A, in mitophagy, indicating that these lectins can function broadly to control target selectivity in autophagy (Soleimanpour et al., 2014).

Here, we integrated time course RNA-seq data and quantitative proteomics to identify relevant *CLEC12A*-dependent protein interactions that are involved in antibacterial autophagy. We identified an E3 ligase complex including KLHL9, NEDD8, and KLHL13 that interacts with *CLEC12A* and is required for antibacterial autophagy. Transcriptomic data suggested that the association of *CLEC12A* with this E3 ligase complex is important for the *CLEC12A*-dependent response to bacterial infection. Virulence factors produced by some bacteria are known to directly target neddylated cullin-associated ubiquitin ligase activity, suggesting that these proteins might be broadly involved in inhibiting bacterial pathogenesis (Jubelin et al., 2010). Additionally, KLHL9 has been previously associated with early onset autosomal dominant distal myopathy, a disease in which alterations to the autophagy pathway are thought to contribute to pathophysiology (Cirak et al., 2010). Taken together, these data identify a role for the recently evolved substrate adapters KLHL9 and KLHL13 as well as NEDD8 in the restriction of bacterial infection.

Previous studies have suggested roles for the E3 ligases SMURF1, LRSAM1, and PARKIN in pathogen-specific autophagy (Huett et al., 2012; Manzanillo et al., 2013; Orvedahl et al., 2011). Recent studies have also highlighted roles for other E3 ubiquitin ligases in the control of bulk autophagy. These include a role for RNF5 in regulating the levels of ATG4B (Kuang et al., 2012), a role for Cullin-5 and Cullin-4 in autophagy regulation through AMBRA1 (Antonoli et al., 2014), and a role for RNF216 in autophagy regulation through Beclin1 (Xu et al., 2014). Additionally, several members of the TRIM family of proteins can act as a platform for assembly of the autophagy machinery (Mandell et al., 2014). Expression of RNF5 and RNF216 were also shown to increase pathogen susceptibility through autophagy (Kuang et al., 2012; Xu et al., 2014). Taken together with our data, these findings demonstrate that E3 ubiquitin ligases play an important role in regulating autophagy at different stages of the pathway. Specifically, E3 ubiquitin ligases create selectivity in autophagy regulation by recognizing and integrating specific signals from pathogens and cellular states. Utilizing E3 ligases to tightly control the response to pathogens is likely a highly conserved mechanism of innate defense (Pollier et al., 2013).

Here, we use integrated genomics to demonstrate a role for *CLEC12A* in antibacterial autophagy and identify an E3 ligase complex that provides insight into the selectivity of pathogen degradation. Our in vivo results highlight a strong role for *CLEC12A* in the restriction of microbial replication. It is possible given this dramatic effect that *CLEC12A* functions not only in antibacterial autophagy but in other pathogen defense pathways



as well. This study highlights how perturbational profiling can be used to study pathways underlying immunity and pathogen defense and illustrates the potential of combining whole-genome experimental data sets to understand functional gene interactions within a relevant pathway.

## EXPERIMENTAL PROCEDURES

### Isolation of PBMCs and Perturbation

For perturbational profiling, blood was collected after written informed consent (or waiver as approved by the institutional review board) from healthy volunteers at Radboud University Nijmegen Medical Centre (RUNMC). The study was approved by the institutional review boards and was performed in accordance with the Declaration of Helsinki. Separation and stimulation of PBMCs from healthy individuals was performed as described previously (Netea et al., 2004). Microarray hybridization and genotyping of volunteers was performed as previously described (Smeekens et al., 2013). Factorial design analysis was performed as described previously (Cadwell et al., 2010). See the [Supplemental Experimental Procedures](#) for further details.

### Antibacterial Autophagy Assays

*S. Typhimurium* infections of HeLa cells and gentamicin protection assays were performed as previously described (Huett et al., 2012). For entry assays, cells were infected for 20 min, washed, fixed in 4% paraformaldehyde for 15 min at room temperature, and stained as described below. For antibacterial autophagy assays in siRNA-treated cells, RNAi knockdown for 48 hr was performed as described above on HeLa cells plated on glass coverslips and infected as above. Infections of BMDMs with *Listeria* were performed with strain EGDe. See the [Supplemental Experimental Procedures](#) for further details.

### Classical Autophagy Assay

Autophagy was induced in HeLa cells by treatment for 4 hr with 100 nM Torin-1, 10  $\mu\text{g ml}^{-1}$  of E64d-Pepstatin A (Sigma-Aldrich), or mock treatment with DMSO. Cells were treated with siRNA for 48 hr as described above and then autophagy was induced followed by cell lysis (25 mM Tris [pH 7.5], 0.5% NP-40, 150 mM NaCl, and protease inhibitors [Roche]). Western blotting to demonstrate LC3 lipidation was performed after equalization of protein amounts and SDS-PAGE on an AnyKD polyacrylamide gel (Bio-Rad). Following transfer to Immobilon-P membranes (Millipore), detection was performed using rabbit anti-LC3 primary (Sigma-Aldrich), mouse anti-actin (Sigma-Aldrich), and appropriate fluorescent secondary antibodies (LI-COR Biosciences) as previously described.

### Gentamicin Protection Assay

Bacterial survival assays were performed as previously described (Huett et al., 2012; Lassen et al., 2014). See the [Supplemental Experimental Procedures](#) for further details.

### CRISPR ATG16L1 Knockout and Complementation

The second exon of *ATG16L1* was targeted in HeLa cells using the px330 plasmid CRISPR system as described (Ran et al., 2013). See the [Supplemental Experimental Procedures](#) for further details.

### In Vivo Salmonella Infection

Mice were maintained in specific-pathogen-free facilities at Massachusetts General Hospital. All animal studies were conducted under protocols approved by the Subcommittee on Research Animal Care (SRAC) at Massachusetts General Hospital. *Clec12a*<sup>-/-</sup> mice were obtained from the laboratory of G.D.B. at the University of Aberdeen. *Clec12a*<sup>-/-</sup> mice were produced by conventional gene targeting of exons 1–4 on a C57BL/6 background (P. Redelinghuys, A. Augello, R.A. Drummond, J.-M. Levesque, S. Vautier, D.M. Reid, B. Kerscher, J.A. Taylor, P.A. Nigrovic, J. Wright, G.I. Murray, L.J. Hocking, M.J.G. Fernandes, C. De Bari, I.B. McInnes, and G.D.B., unpublished data). Mice were born at Mendelian ratios. Protocols for bacterial growth and infection were performed as previously described, with slight modification (Barthel

et al., 2003; Conway et al., 2013). See the [Supplemental Experimental Procedures](#) for further details.

### Quantitative Proteomics

Quantitative proteomics using iTRAQ labeling of peptides was performed as described previously (Lassen et al., 2014; Mertins et al., 2014). See the [Supplemental Experimental Procedures](#) for further details.

### Osmotic Stress Assay

HeLa cells were reverse-transfected with plasmid expressing GFP-CLEC12A on glass coverslips in 12-well plates the night before the assay. Osmotic lysis of endosomes was accomplished by exposing cells to hypertonic medium (0.5 M sucrose in PBS with 10% PEG100) for 10 min. Cells were washed twice with 1× PBS and then incubated in 60% PBS for 3 min as described previously (Thurston et al., 2012). Cells were returned to complete medium for 20 min and then fixed with ice-cold methanol.

### ACCESSION NUMBERS

The accession number for the PBMC stimulation experiments reported in this paper is GEO: GSE68765. The accession number for the BMDM RNA-seq experiments reported in this paper is GEO: GSE69132.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.05.045>.

### AUTHOR CONTRIBUTIONS

J.B., K.G.L., H.B.J., L.A.B., R.J.H., J.M.T., S.-Y.K., E.J.V., L.F., M.O., and V.K. performed experiments. K.G.L., L.A.B., G.G., and A.N. performed data analysis. M.S., S.A.C., L.A.B.J., J.M.V., M.J.D., M.G.N., G.D.B., C.W., and R.J.X. directed the research. G.D.B. supplied critical reagents. J.B., K.G.L., and R.J.X. wrote the paper.

### ACKNOWLEDGMENTS

We thank Mathieu Platteel (UMCG) for microarray experiments and Natalia Nedelsky for editorial assistance. R.J.X. was supported by the Leona M. and Harry B. Helmsley Charitable Trust, the Crohn's and Colitis Foundation of America, and grants AI109725 and DK097485 from the NIH. C.W. is supported by funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Advanced grant agreement 2012-322698. M.G.N. was supported by an ERC Consolidator Grant (no. 310372). G.D.B. was supported by the Wellcome Trust. H.B.J. was supported by a clinical fellowship from Alberta Innovates-Health Solutions (AIHS), Alberta, Canada.

Received: January 12, 2015

Revised: May 4, 2015

Accepted: May 26, 2015

Published: June 18, 2015

### REFERENCES

- Anand, P.K., Tait, S.W., Lamkanfi, M., Amer, A.O., Nunez, G., Pagès, G., Pouyssegur, J., McGargill, M.A., Green, D.R., and Kanneganti, T.D. (2011). TLR2 and RIP2 pathways mediate autophagy of *Listeria monocytogenes* via extracellular signal-regulated kinase (ERK) activation. *J. Biol. Chem.* 286, 42981–42991.
- Antonoli, M., Albiero, F., Nazio, F., Vescovo, T., Perdomo, A.B., Corazzari, M., Marsella, C., Piselli, P., Gretzmeier, C., Dengjel, J., et al. (2014). AMBRA1 interplay with cullin E3 ubiquitin ligases regulates autophagy dynamics. *Dev. Cell* 31, 734–746.

- Barreiro, L.B., Tailleux, L., Pai, A.A., Gicquel, B., Marioni, J.C., and Gilad, Y. (2012). Deciphering the genetic architecture of variation in the immune response to *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA* **109**, 1204–1209.
- Barthel, M., Hapfelmeier, S., Quintanilla-Martínez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Rüssmann, H., and Hardt, W.D. (2003). Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect. Immun.* **71**, 2839–2858.
- Basha, O., Tirman, S., Eluk, A., and Yeger-Lotem, E. (2013). Response-Net2.0: Revealing signaling and regulatory pathways connecting your proteins and genes—now with human data. *Nucleic Acids Res.* **41**, W198–W203.
- Behrends, C., Sowa, M.E., Gygi, S.P., and Harper, J.W. (2010). Network organization of the human autophagy system. *Nature* **466**, 68–76.
- Benjamin, J.L., Sumpster, R., Jr., Levine, B., and Hooper, L.V. (2013). Intestinal epithelial autophagy is essential for host defense against invasive bacteria. *Cell Host Microbe* **13**, 723–734.
- Birmingham, C.L., Smith, A.C., Bakowski, M.A., Yoshimori, T., and Brumell, J.H. (2006). Autophagy controls *Salmonella* infection in response to damage to the *Salmonella*-containing vacuole. *J. Biol. Chem.* **281**, 11374–11383.
- Birmingham, C.L., Canadien, V., Gouin, E., Troy, E.B., Yoshimori, T., Cosart, P., Higgins, D.E., and Brumell, J.H. (2007). *Listeria monocytogenes* evades killing by autophagy during colonization of host cells. *Autophagy* **3**, 442–451.
- Cadwell, K., Patel, K.K., Maloney, N.S., Liu, T.C., Ng, A.C., Storer, C.E., Head, R.D., Xavier, R., Stappenbeck, T.S., and Virgin, H.W. (2010). Virus-plus-susceptibility gene interaction determines Crohn's disease gene *Atg16L1* phenotypes in intestine. *Cell* **141**, 1135–1145.
- Castillo, E.F., Dekonenko, A., Arko-Mensah, J., Mandell, M.A., Dupont, N., Jiang, S., Delgado-Vargas, M., Timmins, G.S., Bhattacharya, D., Yang, H., et al. (2012). Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. *Proc. Natl. Acad. Sci. USA* **109**, E3168–E3176.
- Cemma, M., Kim, P.K., and Brumell, J.H. (2011). The ubiquitin-binding adaptor proteins p62/SQSTM1 and NDP52 are recruited independently to bacteria-associated microdomains to target *Salmonella* to the autophagy pathway. *Autophagy* **7**, 341–345.
- Chen, J.C., Alvarez, M.J., Talos, F., Dhruv, H., Rieckhof, G.E., Iyer, A., Diefes, K.L., Aldape, K., Berens, M., Shen, M.M., and Califano, A. (2014). Identification of causal genetic drivers of human disease through systems-level analysis of regulatory networks. *Cell* **159**, 402–414.
- Cirak, S., von Deimling, F., Sachdev, S., Errington, W.J., Herrmann, R., Bönne-mann, C., Brockmann, K., Hinderlich, S., Lindner, T.H., Steinbrecher, A., et al. (2010). Kelch-like homologue 9 mutation is associated with an early onset autosomal dominant distal myopathy. *Brain* **133**, 2123–2135.
- Conway, K.L., Kuballa, P., Song, J.H., Patel, K.K., Castoreno, A.B., Yilmaz, O.H., Jijon, H.B., Zhang, M., Aldrich, L.N., Villablanca, E.J., et al. (2013). *Atg16L1* is required for autophagy in intestinal epithelial cells and protection of mice from *Salmonella* infection. *Gastroenterology* **145**, 1347–1357.
- Crow, A., Hughes, R.K., Taieb, F., Oswald, E., and Banfield, M.J. (2012). The molecular basis of ubiquitin-like protein NEDD8 deamidation by the bacterial effector protein Cif. *Proc. Natl. Acad. Sci. USA* **109**, E1830–E1838.
- Dambuz, I.M., and Brown, G.D. (2015). C-type lectins in immunity: recent developments. *Curr. Opin. Immunol.* **32**, 21–27.
- de Grado, M., Rosenberger, C.M., Gauthier, A., Vallance, B.A., and Finlay, B.B. (2001). Enteropathogenic *Escherichia coli* infection induces expression of the early growth response factor by activating mitogen-activated protein kinase cascades in epithelial cells. *Infect. Immun.* **69**, 6217–6224.
- Delgado, M.A., Elmaoued, R.A., Davis, A.S., Kyei, G., and Deretic, V. (2008). Toll-like receptors control autophagy. *EMBO J.* **27**, 1110–1121.
- Deretic, V., Saitoh, T., and Akira, S. (2013). Autophagy in infection, inflammation and immunity. *Nat. Rev. Immunol.* **13**, 722–737.
- Drummond, R.A., and Brown, G.D. (2013). Signalling C-type lectins in antimicrobial immunity. *PLoS Pathog.* **9**, e1003417.
- Ernst, J., and Bar-Joseph, Z. (2006). STEM: a tool for the analysis of short time series gene expression data. *BMC Bioinformatics* **7**, 191.
- Franke, A., McGovern, D.P., Barrett, J.C., Wang, K., Radford-Smith, G.L., Ahmad, T., Lees, C.W., Balschun, T., Lee, J., Roberts, R., et al. (2010). Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* **42**, 1118–1125.
- Gagneur, J., Stegle, O., Zhu, C., Jakob, P., Tekkedil, M.M., Aiyar, R.S., Schuon, A.K., Pe'er, D., and Steinmetz, L.M. (2013). Genotype-environment interactions reveal causal pathways that mediate genetic effects on phenotype. *PLoS Genet.* **9**, e1003803.
- Gat-Viks, I., Chevrier, N., Wilentzik, R., Eisenhaure, T., Raychowdhury, R., Steuerman, Y., Shalek, A.K., Hacohen, N., Amit, I., and Regev, A. (2013). Deciphering molecular circuits from genetic variation underlying transcriptional responsiveness to stimuli. *Nat. Biotechnol.* **31**, 342–349.
- Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., and Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* **119**, 753–766.
- Huett, A., Heath, R.J., Begun, J., Sassi, S.O., Baxt, L.A., Vyas, J.M., Goldberg, M.B., and Xavier, R.J. (2012). The LRR and RING domain protein LRSAM1 is an E3 ligase crucial for ubiquitin-dependent autophagy of intracellular *Salmonella* Typhimurium. *Cell Host Microbe* **12**, 778–790.
- Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cézard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M., et al. (2001). Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411**, 599–603.
- Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M., et al. (2003). Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.* **278**, 5509–5512.
- Jostins, L., Ripke, S., Weersma, R.K., Duerr, R.H., McGovern, D.P., Hui, K.Y., Lee, J.C., Schumm, L.P., Sharma, Y., Anderson, C.A., et al.; International IBD Genetics Consortium (IBDGC) (2012). Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* **491**, 119–124.
- Jubelin, G., Taieb, F., Duda, D.M., Hsu, Y., Samba-Louaka, A., Nobe, R., Penary, M., Watrin, C., Nougayrède, J.P., Schulman, B.A., et al. (2010). Pathogenic bacteria target NEDD8-conjugated cullins to hijack host-cell signaling pathways. *PLoS Pathog.* **6**, e1001128.
- Knights, D., Lassen, K.G., and Xavier, R.J. (2013). Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome. *Gut* **62**, 1505–1510.
- Kuang, E., Okumura, C.Y., Sheffy-Levin, S., Varsano, T., Shu, V.C., Qi, J., Niesman, I.R., Yang, H.J., López-Otín, C., Yang, W.Y., et al. (2012). Regulation of ATG4B stability by RNF5 limits basal levels of autophagy and influences susceptibility to bacterial infection. *PLoS Genet.* **8**, e1003007.
- Lahoud, M.H., Proietto, A.I., Ahmet, F., Kitsoulis, S., Eidsmo, L., Wu, L., Sathe, P., Pietersz, S., Chang, H.W., Walker, I.D., et al. (2009). The C-type lectin Clec12A present on mouse and human dendritic cells can serve as a target for antigen delivery and enhancement of antibody responses. *J. Immunol.* **182**, 7587–7594.
- Lassen, K.G., Kuballa, P., Conway, K.L., Patel, K.K., Becker, C.E., Peloquin, J.M., Villablanca, E.J., Norman, J.M., Liu, T.C., Heath, R.J., et al. (2014). *Atg16L1* T300A variant decreases selective autophagy resulting in altered cytokine signaling and decreased antibacterial defense. *Proc. Natl. Acad. Sci. USA* **111**, 7741–7746.
- Lhocine, N., Arena, E.T., Bomme, P., Ubelmann, F., Prévost, M.C., Robine, S., and Sansonetti, P.J. (2015). Apical invasion of intestinal epithelial cells by

- Salmonella typhimurium requires villin to remodel the brush border actin cytoskeleton. *Cell Host Microbe* 17, 164–177.
- Mandell, M.A., Jain, A., Arko-Mensah, J., Chauhan, S., Kimura, T., Dinkins, C., Silvestri, G., Münch, J., Kirchhoff, F., Simonsen, A., et al. (2014). TRIM proteins regulate autophagy and can target autophagic substrates by direct recognition. *Dev. Cell* 30, 394–409.
- Manzanillo, P.S., Ayres, J.S., Watson, R.O., Collins, A.C., Souza, G., Rae, C.S., Schneider, D.S., Nakamura, K., Shiloh, M.U., and Cox, J.S. (2013). The ubiquitin ligase parkin mediates resistance to intracellular pathogens. *Nature* 501, 512–516.
- Marshall, A.S., Willment, J.A., Pyz, E., Dennehy, K.M., Reid, D.M., Dri, P., Gordon, S., Wong, S.Y., and Brown, G.D. (2006). Human MICL (CLEC12A) is differentially glycosylated and is down-regulated following cellular activation. *Eur. J. Immunol.* 36, 2159–2169.
- McDermott, J.E., Archuleta, M., Thrall, B.D., Adkins, J.N., and Waters, K.M. (2011). Controlling the response: predictive modeling of a highly central, pathogen-targeted core response module in macrophage activation. *PLoS ONE* 6, e14673.
- Mertins, P., Yang, F., Liu, T., Mani, D.R., Petyuk, V.A., Gillette, M.A., Clauser, K.R., Qiao, J.W., Gritsenko, M.A., Moore, R.J., et al. (2014). Ischemia in tumors induces early and sustained phosphorylation changes in stress kinase pathways but does not affect global protein levels. *Mol. Cell. Proteomics* 13, 1690–1704.
- Munafó, D.B., and Colombo, M.I. (2002). Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic* 3, 472–482.
- Murthy, A., Li, Y., Peng, I., Reichelt, M., Katakam, A.K., Noubade, R., Roose-Girma, M., DeVoss, J., Diehl, L., Graham, R.R., and van Lookeren Campagne, M. (2014). A Crohn's disease variant in Atg16l1 enhances its degradation by caspase 3. *Nature* 506, 456–462.
- Netea, M.G., Kullberg, B.J., de Jong, D.J., Franke, B., Sprong, T., Naber, T.H., Drenth, J.P., and Van der Meer, J.W. (2004). NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn's disease. *Eur. J. Immunol.* 34, 2052–2059.
- Neumann, K., Castiñeiras-Vilariño, M., Höckendorf, U., Hanneschläger, N., Lemeer, S., Kupka, D., Meyermann, S., Lech, M., Anders, H.J., Kuster, B., et al. (2014). Clec12a is an inhibitory receptor for uric acid crystals that regulates inflammation in response to cell death. *Immunity* 40, 389–399.
- Ogawa, M., Yoshimori, T., Suzuki, T., Sagara, H., Mizushima, N., and Sasaki, C. (2005). Escape of intracellular Shigella from autophagy. *Science* 307, 727–731.
- Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., et al. (2001). A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411, 603–606.
- Orvedahl, A., Sumpter, R., Jr., Xiao, G., Ng, A., Zou, Z., Tang, Y., Narimatsu, M., Gilpin, C., Sun, Q., Roth, M., et al. (2011). Image-based genome-wide siRNA screen identifies selective autophagy factors. *Nature* 480, 113–117.
- Osorio, F., and Reis e Sousa, C. (2011). Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* 34, 651–664.
- Plantinga, T.S., Crisan, T.O., Oosting, M., van de Veerdonk, F.L., de Jong, D.J., Philpott, D.J., van der Meer, J.W., Girardin, S.E., Joosten, L.A., and Netea, M.G. (2011). Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. *Gut* 60, 1229–1235.
- Pollier, J., Moses, T., González-Guzmán, M., De Geyter, N., Lippens, S., Vanden Bossche, R., Marhavý, P., Kremer, A., Morreel, K., Guérin, C.J., et al. (2013). The protein quality control system manages plant defence compound synthesis. *Nature* 504, 148–152.
- Ran, F.A., Hsu, P.D., Lin, C.Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y., and Zhang, F. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154, 1380–1389.
- Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., et al. (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat. Genet.* 39, 596–604.
- Sadaghian Sadabad, M., Regeling, A., de Goffau, M.C., Blokzijl, T., Weersma, R.K., Penders, J., Faber, K.N., Harmsen, H.J., and Dijkstra, G. (2014). The ATG16L1-T300A allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn's disease patients. *Gut*, Published online September 24, 2014. <http://dx.doi.org/10.1136/gutjnl-2014-307289>.
- Schulz, M.H., Devanny, W.E., Gitter, A., Zhong, S., Ernst, J., and Bar-Joseph, Z. (2012). DREM 2.0: Improved reconstruction of dynamic regulatory networks from time-series expression data. *BMC Syst. Biol.* 6, 104.
- Shan, J., Balasubramanian, M.N., Donelan, W., Fu, L., Hayner, J., Lopez, M.C., Baker, H.V., and Kilberg, M.S. (2014). A mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)-dependent transcriptional program controls activation of the early growth response 1 (EGR1) gene during amino acid limitation. *J. Biol. Chem.* 289, 24665–24679.
- Shin, D.M., Yuk, J.M., Lee, H.M., Lee, S.H., Son, J.W., Harding, C.V., Kim, J.M., Modlin, R.L., and Jo, E.K. (2010). Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cell. Microbiol.* 12, 1648–1665.
- Silver, M., Chen, P., Li, R., Cheng, C.Y., Wong, T.Y., Tai, E.S., Teo, Y.Y., and Montana, G. (2013). Pathways-driven sparse regression identifies pathways and genes associated with high-density lipoprotein cholesterol in two Asian cohorts. *PLoS Genet.* 9, e1003939.
- Singh, S.B., Davis, A.S., Taylor, G.A., and Deretic, V. (2006). Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 313, 1438–1441.
- Smeekens, S.P., Ng, A., Kumar, V., Johnson, M.D., Plantinga, T.S., van Dieën, C., Arts, P., Verweil, E.T., Gresnigt, M.S., Fransen, K., et al. (2013). Functional genomics identifies type I interferon pathway as central for host defense against *Candida albicans*. *Nat. Commun.* 4, 1342.
- Smith, E.N., and Kruglyak, L. (2008). Gene-environment interaction in yeast gene expression. *PLoS Biol.* 6, e83.
- Soleimanpour, S.A., Gupta, A., Bakay, M., Ferrari, A.M., Groff, D.N., Fadista, J., Spruce, L.A., Kushner, J.A., Groop, L., Seeholzer, S.H., et al. (2014). The diabetes susceptibility gene Clec16a regulates mitophagy. *Cell* 157, 1577–1590.
- Tattoli, I., Sorbara, M.T., Vuckovic, D., Ling, A., Soares, F., Carneiro, L.A., Yang, C., Emili, A., Philpott, D.J., and Girardin, S.E. (2012). Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. *Cell Host Microbe* 11, 563–575.
- Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J. Biol. Chem.* 284, 8023–8032.
- Thurston, T.L., Ryzhakov, G., Bloor, S., von Muhlen, N., and Randow, F. (2009). The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. *Nat. Immunol.* 10, 1215–1221.
- Thurston, T.L., Wandel, M.P., von Muhlen, N., Foeglein, A., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. *Nature* 482, 414–418.
- Watanabe, T., Asano, N., Murray, P.J., Ozato, K., Tailor, P., Fuss, I.J., Kitani, A., and Strober, W. (2008). Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. *J. Clin. Invest.* 118, 545–559.
- Wild, P., Farhan, H., McEwan, D.G., Wagner, S., Rogov, V.V., Brady, N.R., Richter, B., Korac, J., Waidmann, O., Choudhary, C., et al. (2011).

Phosphorylation of the autophagy receptor optineurin restricts *Salmonella* growth. *Science* 333, 228–233.

Xu, C., Feng, K., Zhao, X., Huang, S., Cheng, Y., Qian, L., Wang, Y., Sun, H., Jin, M., Chuang, T.H., and Zhang, Y. (2014). Regulation of autophagy by E3 ubiquitin ligase RNF216 through BECN1 ubiquitination. *Autophagy* 10, 2239–2250.

Yang, Z., and Klionsky, D.J. (2010). Eaten alive: a history of macroautophagy. *Nat. Cell Biol.* 12, 814–822.

Zelenay, S., Keller, A.M., Whitney, P.G., Schraml, B.U., Deddouche, S., Rogers, N.C., Schulz, O., Sancho, D., and Reis e Sousa, C. (2012). The dendritic cell receptor DNGR-1 controls endocytic handling of necrotic cell antigens to favor cross-priming of CTLs in virus-infected mice. *J. Clin. Invest.* 122, 1615–1627.

Zhang, B., Gaiteri, C., Bodea, L.G., Wang, Z., McElwee, J., Podtelezhnikov, A.A., Zhang, C., Xie, T., Tran, L., Dobrin, R., et al. (2013). Integrated systems approach identifies genetic nodes and networks in late-onset Alzheimer's disease. *Cell* 153, 707–720.